

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

DAFTARY, *et al.*

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For: NON-PEGYLATED LONG-CIRCULATING LIPOSOMES

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Examiner: Gollamudi KISHORE

Attorney Docket No.: 067080-0003

DECLARATION OF DR. GAUTAM VINOD DAFTARY

FILED VIA EFS

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

Sir:

1. I, Dr. Gautam Vinod Daftary, am an inventor of the invention claimed in the present U.S. application, 10/748,094.
2. I have a degree in Medicine.
3. I founded Research and Development Departments in Bharat Serums and Vaccines Ltd. guiding the Research group in the area of Novel Drug Delivery Products, Equine Products, Biotechnology Products, Diagnostic Products, Fertility Hormone Products and also Stem Cells. Majority of the products commercialized by Bharat Serums is based on technology developed in-house.

4. I have more than 20 years of experience in the field of Drug Development and more than 15 years of experience in the field of liposomes.
5. The Liposomal Doxorubicin product, which is a subject matter of this Patent Application has already been commercialized in India. This product has been well accepted because of the better safety profile and a provision of increased dosage administration without experiencing the Hand-Foot Syndrome of Pegylated Liposomal Doxorubicin and cardiotoxicity of Adriamycin.
6. I was also responsible for development of Liposomal Amphotericin B which has also been manufactured on a commercial scale.
7. I was also responsible for successful development of Generic Version of Pegylated Liposomal Doxorubicin which is being commercialized.
8. In addition to the above liposomal products, I have exposure and experience in development on many other liposomal products such as Irinotecan Liposomes, Polymyxin B Sulfate Liposomes etc.
9. I am an inventor for number of patents that have been granted to Bharat Serums in different countries. The details on the patent applications have been listed in the Annexure 1.
10. I am one of the inventors of the application and am also familiar with the office action dated March 9, 2011 as well as the cited references: Hong (Clinical Cancer Research, 1999); Janoff (US 4,880,635); Papahadjopoulos (US 4,235,871); Barenholz (US 5,316,771); Slater (US 6,355,268); Forssen (US 5,714, 163) and Clerc (US 5,939,096).
11. I believe that claim 69 is not indefinite to a person skilled in the art who is well conversant with the terminology of circulation time of a therapeutic or diagnostic agent (active material). One skilled in the art in the field of liposome preparation and the study

of liposome pharmacokinetics would understand the term “liposomes have a blood circulation half life of at least 25 times longer than conventional non-liposomal formulations when tested in Swiss albino mice at equivalent doses.” It is a common practice to check and compare the properties of the liposomes with the free drug. Even the references quoted in the Office Action follow the same practice (*See for example Slater et al. US 6,35,5268*).

12. I do not want to contradict the inference drawn by the Examiner that “*...it is natural for the liposome formulation to have a longer circulation times because liposomes are sustained release vehicles...*” However, the present invention magnifies the difference multifold. This property of the composition having multifold difference in the circulation time makes it suitable for use in Clinical Practice. If one were to go logically by the above inference, there should have been many more liposomal products in the world market than there actually are based on the Examiner’s erroneous belief that liposomes are sustained release vehicles and putting in the liposomal vehicle would automatically increase the circulation time.
13. Conventional liposomes (without pegylated phospholipids) are opsonized by plasma proteins and trapped by the reticuloendothelial system (“RES”). Surprisingly, the liposomes of the present invention (without the use of pegylated phospholipids) show slower RES uptake and hence, longer circulation time.
14. The specification provides Examples showing how to make the liposomes via the claimed method and how to load them with Doxorubicin. The Examples further show how these liposomes were tested against nonliposomal Doxorubicin (ADRIAMYCIN). Also, the specification provides test results indicating that liposomes made by the present invention have a blood circulation half life of at least 25 times longer than conventional non-liposomal formulations when tested in Swiss albino mice at equivalent doses.

15. Comparing a liposomal formulation containing a therapeutic agent against the same therapeutic agent not in a liposome is typical for the industry and such tests are conventionally accepted for studying liposome circulation time as well as their ability to carry and deliver their contents.
16. The purpose of preparing a liposome is to entrap the active ingredient in it. There is no purpose to inject a blank liposome in a human system, and no purpose in studying its circulation time without an active ingredient. The liposome stability in the presence of the entrapped therapeutic or diagnostic agent (active material) is the most relevant use of the liposome. The whole purpose of using the liposome is to keep the therapeutic or diagnostic agent (active material) in circulation for a certain time. Therefore, if one wants to find out how much the circulation time of a liposome has increased, one has to compare it with the circulation time of the therapeutic or diagnostic agent (active material) itself as it is normally injected (which is what is referred to as a "non-liposomal therapeutic or diagnostic agent (active material)." That is the basic standard by which measurements of liposome stability in blood are measured during circulation. As explained in more detail below, there is nothing like a standard liposome as the Examiner suggests, and as such, the industry does not compare circulations times to some standardized liposome, but rather compares to the therapeutic or diagnostic agent administered alone. Further, the measure of advantage of increased retention time of the liposome encapsulated drug in blood is reflected truly if compared with retention time of the drug in blood as such. $T_{1/2}$ values are not absolute values, they are dependent on many factors of the liposomal compositions, structure and the entrapped active matter in it and hence are always comparative values.
17. Even Slater (quoted in Office Action) compares liposome entrapped MPE Camptothecin with the "drug administered in free form" for therapeutic efficacy as well as for studying MTD (Maximum Tolerated Dose) and LED (Lowest Effective Dose).

18. There is no standardized liposome (non-pegylated or pegylated liposome), in part because liposome properties vary according to their contents and their method of preparation. Liposomes and their behavior (stability while in circulation in the body (i.e. length of circulation time); ability to carry certain active ingredients without leaking, storage length, ability to entrap certain therapeutics efficiently, etc.) all depend on multiple factors, and all of these factors can work together, or against each other, or in some cases, work together to provide synergistic benefits. Some of these factors include: the liposome components (i.e. the phospholipids, lipids, sterol, PEG, etc. and their amounts and ratios used); the size of the liposomes; the solutions used to make and load the liposomes; the conditions followed for making or loading the liposome; and the amount of the solutions used, etc.
19. The art of liposome manufacture is unpredictable because so many factors can be chosen and used in the manufacture, and hence the industry does not recognize a "standard" liposome. For example, if two groups made a liposome composed of the same phospholipids and same sterols, the characteristics of these liposomes most likely will differ if they are made by a different process and especially if they were made with different solutions and different amounts of solutions.
20. The findings of the present invention show that the liposomal therapeutic or diagnostic agent (active material) - that is entrapped in the liposome of the present invention, has considerable improvement over the non-liposomal drug in terms of circulation time, i.e. $T_{1/2}$ without losing the efficacy (See Table 2 and Table 3 of the specification). Thus, the liposome of the present invention not only itself has prolonged circulation in the blood but it is also efficient in keeping the drug entrapped inside it to effect gradual release of the entrapped drug in smaller doses into blood during/throughout this prolonged period for action at the appropriate site. Its effect in breast tumor reduction over that of the currently marketed pegylated liposomal formulation (CAELYX) has been found comparable (see Table 2 of the specification). The extent of this improvement is based on the increased circulation time, i.e. $T_{1/2}$ increased by more than 25 times than that of

circulation time of the non-liposomal therapeutic agent (active material) in the form as commonly used at the time of invention. Even Slater (prior art cited in the Office Action) shows in Table 5 that $T_{1/2}$ of the drug entrapped in the pegylated liposome is 32 -36 times that of the free drug. $T_{1/2}$ of the drug entrapped in non-pegylated liposome of Example II in the present invention is 48 times that of the free drug (see Table 1 of the instant specification). One skilled in the art would understand that this is a normal comparison and would clearly understand the metes and bounds of the invention and what the applicants have defined as their invention.

21. It is my belief that it is the structure of the liposome (which was achieved by combining the claimed liposome components with the claimed solutions in the claimed process) that makes the resulting liposomes long circulating (i.e. hold the therapeutic or diagnostic agent entrapped in it for longer time). Prior to the present invention, others altered the structure by adding polyethylene glycol (PEG) derivatised phospholipids. These PEGylated liposomes, because of the polymer coatings on the bilayers of the liposome membranes, have been shown to hold the therapeutic or diagnostic agent for a longer time than non-pegylated liposomes by avoiding RES trapping. In contrast to using PEG, the present invention has achieved longer circulation by altering the liposome's structure by using the claimed method of manufacture combined with the specific claimed liposome components and solutions used in the process of making.
22. The claimed process of manufacture recites mainly four groups of elements all necessary for the desired result of a non-pegylated long circulating liposome: 1) the components of the liposomes consisting of certain phospholipids i.e. DSPC, HSPC, or mixtures thereof (no pegylated phospholipids) and sterols; 2) the various process steps; 3) the use of certain solutions (i.e. hydration media of ammonium sulfate and sucrose; dialysis buffer of sucrose-histidine); and 4) recited amounts of the certain solutions in conjunction with the liposome components (10-35 ml of the hydration media for each mole of the phospholipid). These (components, steps, solutions and their amounts as claimed) act synergistically to produce unique long circulating non-pegylated liposomes.

23. I believe that the same effect will not be produced in liposomes by using the same quantity of hydration media when the composition of hydration media is different. The composition of hydration media and volume of hydration media used have varying effects not independent of each other. Similarly, what is being hydrated is also part of the reason of deciding on hydration volume. If volume A is used for hydrating DSPC, the same volume may not be good for hydrating DSPC + Cholesterol, or EPC. A person of ordinary skill in the art, being aware of this fact, would not pick up some volume of hydration medium for lipid composition mentioned in one reference and apply it for hydrating another lipid composition mentioned in another reference expecting to get liposomes having properties similar to the liposomes of the present invention. For example, the hydration volume of Janoff would not be considered as Janoff used EPC as his phospholipid, and Janoff used sodium chloride, HEPES and sucrose constituting the hydration medium. Further, Janoff would not be considered by one skilled in the art for combination with Slater, which has Hydrogenated Soya phosphatidylcholine + Cholesterol and PEG-DSPE as phospholipids for hydration, and ammonium sulphate and dextran sulphate-ammonium salt solution as the hydration medium. Similarly, the volume of hydration medium used by Clerc (using weak acid salts) is not suitable for a hydration medium used by Forssen, which uses strong acid salt (ammonium sulphate) and which has no combination of ammonium sulphate and sucrose, as in claim 1. Hence the combinations suggested by the Examiner to show that use of such volume of hydration medium in claim 1 is obvious, is not persuasive and is contrary to how one skilled in the art would review and consider these references.
24. I have collected the facts from the actual examples in the references cited in the Office Action vis-a-vis those claimed in the present invention and have put them in the tabulated form below (see TABLE I, below). The TABLE I shows that sucrose histidine buffer solution for dialysis for removing ammonium sulphate from extraliposomal hydration medium as set for in the claim of the present invention has not been shown or suggested in the references cited by the Examiner. Thus any combination put forward by the Examiner falls short of teaching or suggesting each and every element. In TABLE I, Col.

Col. 1 provides a short name for the reference cited; Col. 2 provides what is being hydrated for making liposome; Col. 3 provides the constituents of the hydration medium; Col. 4 provides the amount of the hydration medium used per mMole of the phospholipid in what is being hydrated, Col. 5 provides whether the reference has any step of removing extra-liposomal hydration medium by dialysis using sucrose-histidine buffer; and Col. 6 provides other peculiar details of the invention.

TABLE I: Hydration step and other differentiating details

<u>Reference</u>	<u>Lipid \rightarrow Phospholipid, sterol etc.</u>	<u>Hydration medium</u>	<u>Volume (ml/mM of Phospholipid) (Rough calculations)</u>	<u>Sucrose-histidine buffer in dialysis*</u>	<u>Other details</u>
Hong	DSPC:Cholesterol - 3:2 + PEG-DSPE	Ammonium sulphate 250 mM pH 5.0	No data	No Step	Particle size is between 65 to 75 nm. 1mg Doxorubicin loading per 10 μ M of phospholipid
Papahadjopoulos	Phosphatidyl-glycerol 10 μ M + phosphatidyl-choline 40 μ M + cholesterol 50 μ M	NaCl 10 mM + 4mM histidine / TES	3ml (1.5ml + 1.5ml) 60 ml/mM of Phospholipids	No Step	Oligolamellar vesicles, particle size between 0.2 to 0.6 μ . Percentage of encapsulation is 34%. Bio-gel A 1.5 agarose column to separate unencapsulated material.
Janoff	EPC 80 μ M	150 mM NaCl + 20mM HEPES + sugar - Aq soln.	2 ml (25ml/mM)	No Step	Unencapsulated material removed by Sephadex G-50 or Ultragel AcA 34 column. Dehydration of liposomes.
Barenholz	100 mg (= 0.132 mM) egg phosphatidyl-choline (mol. wt. 760)	Aqueous ammonium sulphate containing desferal	5ml/0.132mM 38 ml/mM	No Step	
Slater	HSPC + Cholesterol + PEG-DSPE (56.4 :38.3::5.3) Mol.	250mM ammonium sulfate solution; Dextran sulphate ammonium salt solution	3.7g total lipid in 100ml ammonium sulphate solution. 32.6ml/mM of phospholipid	Diafiltration Ammonium sulfate is replaced by sodium chloride or Sucrose	T _{1/2} of drug entrapped in pegylated liposome 32 – 36 times that of free drug
Forssen	DSPC + Cholesterol	Ammonium salt	80.3 mg DSPC/ml	Buffer exchange	

	2:1M 100 mg lipid /ml on hydration	(tartrate, sulphate etc.) solution	(9.8ml/mM)	on Sephadex col.	
Forssen	DSPC + Cholesterol 2:1M 100 mg lipid /ml on hydration	Sucrose 300mM no results		Buffer exchange on Sephadex col.	
Clerc	HSPC; cholesterol - 60:40 M	150 mM weak acid salt -- Na acetate/ 120mM Ca acetate acetate/formate; salts of propanoic, butanoic or pentanoic acids	Vol. adjusted to obtain 10 %w/v lipid conc. i.e. 6g HSPC/100ml, 13.17 ml/mM of Phospholipid	No Step	
Present invention	DSPC and/ or HSPC 1mM	Ammonium sulphate and sucrose	10-35 ml per mM of phospholipid present	Removing ammonium sulphate extra liposomal hydration medium	T _{1/2} of drug entrapped in non-pegylated liposome of Example II is 49 times that of free drug

*For removal of ammonium sulphate from extraliposomal hydration medium

25. The combination of these components, solutions, recited process steps and amounts of certain solutions, unexpectedly provided a long circulating liposome that does not require the presence of pegylated phospholipids to achieve the liposome stability in plasma thereby inducing longer circulation time on parenteral administration. In the case of liposome manufacture, it is well known that one can not simply substitute any step from one method and with another step of a different method especially composition of hydration media, its volume, composition of dialysis buffer etc. and achieve the same end or any predictable result. All of these components act together in an unpredictable manner and thus one can not foresee or predict an outcome or any desired result. Thus, one of ordinary skill in the art would understand that one can not simply mix and match or substitute various components, steps and solutions and achieve any predictable result.
26. Even the references cited in the Office Action show that there is not a standard liposome and further that what may be perceived by the Examiner as minor changes, dramatically effect the properties of the liposome.

27. Forssen shows that changing one small component in the manufacture of liposomes can alter their behavior dramatically. Forssen notes that changing from one ammonium salt to another ammonium salt (e.g. from ammonium tartrate to ammonium sulphate) caused the entrapment procedure to drop in efficiency by over 50%. Thus, contrary to the Examiner's belief, this reference shows that one can not simply interchange solutions, process steps, and liposome components and have any sort of predictable outcome.
28. Determining how much buffer to complete the formation of liposomes is not the same as determining how much buffer is necessary to form non pegylated liposomes having long circulation. In determining how much buffer is needed to form liposomes, one normally uses an excess of hydration media as one wants to have enough hydration media to make sure that the liposomes are formed. One does not want the hydration media to be a rate or process limiting step. Accordingly, the cited references or the general knowledge of one skilled in the art would not motivate one to restrict the amount of hydration media used. Prior to the present invention, no one thought of restricting the hydration media to a certain amount as one of the means in a process to form long circulating non-pegylated liposomes. The researchers concentrated on selecting phospholipids and the composition of the hydration media and studied the properties of the liposomes obtained. After extensive experimentation in the instant invention, it was found that with a selected phospholipids and the chosen hydration media, when the hydration medium is employed at a specified level, and with the chosen dialysis buffer for the removal hydration salt (ammonium sulfate) after the liposomes are formed, the liposomes formed have some specific structure that gives a longer circulation than when the hydration is performed with larger amounts of the hydration media. There is no state of art at the time of the instant invention to teach or suggest that one skilled in the art would know that the liposome formed will have a different property if the hydration medium amount is changed. Therefore, just knowing how much hydration buffer is needed to complete the formation of liposome from the phospholipids taken is not relevant. The hydration amount used in the instant invention is not related to the sufficiency or insufficiency of liposome formation. The role of the quantity of hydration medium was explored along

with exploration of all other elements as noted above in the instant invention to result in a synergistic property of increasing circulation time of the liposome prepared by the entire process. Examples II and XIII provide clear evidence that with other features being the same, the reduction in the volume of the same hydration medium is effective in increasing circulation time.

29. Regarding Janoff, the fact that sucrose or another sugar inhibits leakage of an encapsulated active material from dehydrated liposomes is no indication that it will prevent leakage from the liposomes prior to dehydration or from the rehydrated liposomes after loading with the required drug or other active material. Further, there is no evidence to say that leakage of liposomes has any relevance to produce liposomes having long-circulation time in plasma. I believe that the purpose of the liposomes of the present invention is not to make the liposome impervious to leakage on dehydration, but rather it is to make a liposome that will slowly deliver the drug entrapped in it, and at the same time remain stable over a longer period in the circulating blood at body temperature and Janoff does not suggest such function of his liposomes. Janoff liposomes are dehydrated to a moisture level of up to 2%, under reduced pressure (like lyophilisation) and stored under refrigeration temperature up to 7 days. The nature of the problem to be solved in Janoff is different from that in the present invention. Janoff as a whole does not suggest that use of sucrose in the preparation of liposome would increase its circulation time.
30. The instant invention uses the aqueous hydration medium consisting essentially of ammonium sulfate and sucrose to hydrate phospholipids to form liposomes having long circulation time in plasma, whereas Janoff uses his solutions to have less leakage during dehydration and subsequent hydration of the liposomes, as discussed above. There is no knowledge or evidence to say before this application that presence of sucrose would increase the circulation period. Hong achieved his results without using sucrose. The hydration of phospholipids with a combination of ammonium sulphate and sucrose to form liposomes is for the first time shown in the present invention.

31. There is no need for dehydration or rehydration of liposomes nor any recitation of such a step in Hong, or in the present invention. Accordingly, one of ordinary skill in the art reading Janoff would not be motivated to use sugar along with ammonium sulfate in a hydration medium, especially when the process does not involve dehydration. Janoff does not suggest that sugar addition will increase the circulation time but instead, Janoff's abstract says it is for stabilizing liposomes during hydration and rehydration. There thus would be no motivation to combine Janoff with Hong.
32. Further, there is no reasoning or motivation to pick only sucrose from a laundry list of sugars proffered by Janoff and combine it with ammonium sulfate to achieve a long lasting non-pegylated liposome when Janoff uses sodium chloride and HEPES buffer along with a sugar for an altogether different purpose. To reach the Examiner's conclusion, one would have to speculate in hindsight reasoning. One of ordinary skill in the art would not have expected any increase in circulation time by adding sucrose to the hydration media of Hong upon reading Hong and Janoff, or separating sucrose from sodium chloride and HEPES and combining it with ammonium sulfate before reading the present application.
33. Barenholz does not provide any motivation to practice any other process steps of the claimed invention. Barenholz relates to a procedure for loading amphiphatic drugs and chemicals into liposomes and does not relate to a process of manufacture of long circulating liposomes. Although Barenholz uses an ammonium gradient for loading the liposomes, he does not teach or suggest using an aqueous hydration medium consisting essentially of ammonium sulfate and sucrose. Neither Hong nor Barenholz teach a removal of extraliposomal ammonium sulfate by dialysis using sucrose histidine buffer. Merely using an ammonium gradient for liposome loading does not suggest, teach or motivate one to use a hydration buffer consisting of ammonium sulfate and sucrose to make a liposome.

34. Liposome loading and the process of forming/making liposomes are two entirely different processes. Further, they are not interchangeable and nor are the results of changing different solutions in different steps predictable. One skilled in the art would not look to processes and solutions used to form or make a liposome and believe that they would be interchangeable and work with processes and solutions used to load liposomes once they are formed.
35. Papahadjopoulos does not teach the claim element where the amount of aqueous hydration media used is in the range of 10 to 35 ml for each mmole of phospholipid present. In Papahadjopoulos, the amount of phospholipid used is 50 μ M (cholesterol is 50 μ M) and there is 5ml ether in organic phase. In addition, there is 1.5 ml of aqueous buffered mixture of 10mM sodium chloride solution. Although it is not precisely clear how much water of this aqueous phase remains in viscous gel, applicants assume that the maximum is 1.5 ml. In addition, another 1.5 ml of sodium chloride / histidine / TES buffer is added to the gel when it changes to liposomes. At that stage the maximum water content would be 3 ml. Thus, 3 ml of aqueous phase for 50 μ M of phospholipids converts to 3000 ml per 50 mM, or 60 ml per mM, which is clearly outside of the range of the claimed invention. Even if one were to guess that there would be some loss of water during intermediate evaporation, even if assuming that the aqueous phase may be less than 3 ml (such as 2ml), there would be 2 ml of aqueous phase per 50 μ M of phospholipid. This converts to 2000 ml per 50 mM; or 40 ml per mM which is certainly outside the range of the instant invention.
36. I find that what is taught in Papahadjopoulos regarding the volume of aqueous medium (ml) per millimole of phospholipid has not been calculated correctly in the Office Action. The calculations in the office action calculated volume of aqueous medium (ml) for total lipids (which includes Cholesterol). This in turn would render the calculations erroneous. The claim clearly recited in the present application is that the hydration buffer is used in the range of 10 to 35 ml for each mmole of phospholipid present. The claim does not

recite that other lipids or cholesterol fall within this range. Thus, it is incorrect to take lipids (cholesterol) into account when the claim clearly recites phospholipid.

37. Determining how much buffer to complete the formation of liposomes does not lead one to consider how little is necessary to form a long circulating non pegylated liposome. In addition, it is a combination of all of the claimed process steps i.e. claimed solutions, and claimed liposome components that work synergistically with the amount and make-up of the hydration buffer to achieve the long circulating non-pegylated liposomes. There is always extra liposomal hydration medium remaining after the hydration to form the liposomes. Thus, there is always an excess of the minimum required for the hydration. The claim language of “the amount of aqueous hydration media used is in the range of 10 to 35 ml for each mmole of phospholipid present” defines the total volume of the hydration media used (including this extra/excess hydration media). So the claim is not simply describing the situation where someone merely adds a huge excess of hydration buffer to allow hydration of all of the liposomes but rather, the claim has recited a certain finite amount necessary to achieve hydration but also small enough to provide a tightly packed long circulating non pegylated liposome taking into consideration the other process steps, the make up of the solutions and the make up of the liposomes. Further, the claim also recites that ammonium sulphate is removed from the extraliposomal media in a certain defined step with a certain defined solution: “removing ammonium sulfate from the extra liposomal hydration media by dialysis using a sucrose-histidine buffer solution.”
38. The claimed process works to produce long circulating liposomes based on a combination of all of the elements of the claim (the hydration media, the phospholipid type, the amount of hydration media and the dialysis buffer).
39. One of ordinary skill in the art would not be able to predict the interaction of sugar with the inside membrane of the liposome prepared by using egg lecithin, sodium chloride and HEPES buffer as Janoff teaches, and one could also not be able to predict that sucrose

would interact with internal membrane of a pegylated liposome taught by Hong in the same way. Hence, there would be no motivation to combine Janoff with Hong. One would not be able to predict the interaction of sodium chloride and HEPES buffer and EPC membrane with sugars as used by Janoff to be the same as the interaction of sugars with ammonium sulfate and DSPC and cholesterol in Hong, especially when one process uses dehydration (Janoff) and the other (Hong) does not. A liposome is formed when phospholipid is hydrated, that is when thin film of phospholipid is contacted with water. But the bilayer membrane formed is modified in structure with certain substances included in the hydration medium. To get a particular structure giving a specific performance of liposome other things being equal, hydration medium is critical. Use of NaCl and HEPES buffer along with sucrose in Janoff is critical, and to say that NaCl-HEPES is not critical and only sucrose is critical without any experimental evidence is not persuasive as it is contrary not only to the understanding of one of ordinary skill in the art but also of one skilled in the art of liposome preparation.

40. One would not be motivated to combine Janoff's use of a solution comprising sugar, sodium chloride and HEPES with Hong's lipids of DSPC and Cholesterol, especially since Hong's liposomes were not dehydrated as in Janoff. Although both Hong and Janoff relate to liposomes, there would be no motivation to combine these references because Hong is focusing on comparing the effects of polyethylene glycol (PEG) on doxorubicin liposomes whereas Janoff is focusing on designing a liposome that is stable to dehydration. It is not predictable how different buffers (i.e. Janoff's sugar, sodium chloride and HEPES versus Hong's ammonium sulfate) interact with different liposome components (i.e. Janoff's egg lecithin versus Hong's DSPC and Cholesterol, especially when the process in which these solutions and liposomes are different (Janoff's dehydration versus Hong's lack of dehydration). Thus, one of ordinary skill in the art would not simply consider the volume of hydration media irrespective of difference in the constituents of the hydration media and the process as well as the liposome components.

41. A hydration buffer is used to hydrate and form the liposomes whereas the dialysis buffer is used to remove extra salt. These clearly are two different solutions for two different purposes used at different times. Thus, there is no reason or motivation to take histidine used for hydration of liposomes in Papahadjopoulos and use it in combination with sucrose as a dialysis buffer. When Papahadjopoulos teaches inclusion of histidine, it is for use in a hydration medium used for forming liposomes (and not in dialysis buffer as required by the present claims). I am perplexed as to how the combination of Hong and Papahadjopoulos can be argued to teach, suggest, or motivate the employment of sucrose and histidine buffer for dialysis in removing extraliposomal ammonium sulphate after the liposomes are formed when the process or step of forming a liposome is different than the step of removing excess salt after the liposome is already formed.
42. After reading Slater, one of ordinary skill in the art would not be motivated to try and make a nonpegylated liposome using the method claimed in the present invention as Slater actually teaches away from the claimed method because Slater focuses on the use of vesicle forming lipid derivatized with a hydrophilic polymer (such as pegylated phospholipids) in the manufacture of liposomes and reports that the preferred lipids have PEG as its hydrophilic polymer. Thus, one skilled in the art would not read Slater and be motivated to make a liposome without the use of PEG.
43. More particularly, Slater teaches that lipids used for forming liposomes are derivatized with a hydrophilic polymer. See Col. 2, lines 55-56. Hydrophilic polymers include polyethylene glycol. See Col. 3, lines 22-23. Preferred embodiments utilize polyethylene glycol. See Col 3, lines 24-26. Slater teaches that liposomes formed with lipids derivatized with hydrophilic polymers provide the long circulation time: "The outermost surface coating of hydrophilic polymer chains is effective to provide a liposome with a prolonged circulation time in vivo." See Col 7, lines 43-45. Accordingly, one looking to make a long circulating liposome without using lipids derivatized with polyethylene glycol (a hydrophilic polymer) would not even look to Slater for any motivation or teaching regarding a nonpegylated long circulating liposome

since Slater indicates that use of lipids derivatized with PEG is essential for a long circulating liposome.

44. The Office Action makes one believe that inclusion of sucrose and buffer in the hydrating medium, is the only missing element in Slater, but this does not correspond to facts. Slater lacks the amount of the hydration media used per mMole of phospholipids specified in Claim 1 of present invention. Also Slater uses pegylated phospholipids whereas the present invention is all about use of non-pegylated phospholipids. Further, Slater does not use sucrose-histidine buffer for removing extraliposomal ammonium sulphate as specified in Claim 1 of present invention. Hence, Slater cannot even be considered as a close prior art.
45. Slater does not also teach or suggest removing ammonium sulphate from extra liposomal hydration medium by dialysis using a sucrose-histidine buffer solution as required by the claims. In Slater, the ammonium sulphate and ethanol were removed from the external bulk aqueous phase immediately prior to remote loading of the active agent by hollow fiber tangential flow diafiltration using 10% sucrose solution. This step is clearly different than the claimed process. In example 1 there is a step of removing extra liposomal ammonium sulphate along with ethanol using 10% sucrose solution. In example 4 there is a step involving removal of unentrapped dextran sulphate and ethanol with 8 volume exchanges using 350mM sodium chloride solution followed by 8 volume exchanges using a 10% sucrose solution. Thus, Slater does not teach or suggest the claim element of "removing ammonium sulphate from extra liposomal hydration medium by dialysis using a sucrose-histidine buffer solution." In fact Slater uses 10% sucrose and 10 mMolar histidine for removing un-entrapped drug - not for removing extraliposomal ammonium sulphate before drug loading as in claim 1 of the instant invention. Exchange buffers used before drug loading do not have sucrose-histidine buffer. Just because Slater uses sucrose-histidine buffer in the final liposomal preparation, it cannot said to be obvious to use it in the dialysis step, for even Slater himself does not use it that way.

46. Notwithstanding the deficiencies in Slater and Janoff, one skilled in the art would not be motivated to combine Slater with Janoff. Janoff relates to the use of sugar to protect liposomes during dehydration, whereas Slater is not concerned with dehydration but rather is concerned with retaining the efficacy of the active ingredient topoisomerase inhibitor. The problem with camptothecin and its analogues is that the compounds are susceptible in aqueous environments to hydrolysis at the alpha-hydroxy lactone ring. The lactone ring opens to the carboxylate form of the drug, a form that exhibits little activity against topoisomerase I. Slater uses pegylated phospholipid and makes pegylated liposome to keep the labile therapeutic agent (a topoisomerase inhibitor) intact. One skilled in the art would not consider removing the pegylation used by Slater and instead use the sugars in Janoff when the processes taught by these references are completely different. The condition of liposome formation in Slater requires distribution of polymer on both sides of the liposome bilayer membrane (See Slater col. 2, lines 52 to 58). Slater's process cannot be performed without this condition, which can be done only with pegylated phospholipids.
47. Forssen provides no motivation to combine ammonium sulphate and use it with sucrose in a hydration medium. In Forssen, the scientists achieved poor entrapment when using ammonium sulphate as compared to other ammonium salts. Thus, even though there is mention of an ammonium sulphate for drug loading, there is no teaching or suggestion to use ammonium sulphate in a solution with sucrose to hydrate/form liposomes as required by the claims. Further, one would not even be motivated to use ammonium sulfate in other drug loading procedures because of its poor performance, let alone be motivated to combine with sucrose and use in a different step – liposome hydration.
48. There is no motivation to combine Clerc with Forssen/Slater. Clerc is describing his method as a method of forming liposomes having a higher inside/lower outside pH gradient. The weak acid compounds for loading by his method include ibuprofen, tolmetin, indomethacin, phenylbutazone, meclofenamic acid, piroxycam, ciprofloxacin and nalidixic acid. There is no reason to look at it for increasing circulating time of

liposome without a PEG coating or providing a graft of a hydrophilic polymer chain as mentioned by Clerc. It would not be obvious to one of ordinary skill in the art, to consider only from that portion indicated by examiner in Clerc for making the instant invention when the instant invention does not follow a higher outside/lower inside pH gradient system. The instant invention also does not follow providing a coating or a graft of hydrophilic polymer chain to increase the circulating time of liposomes. There is no motivation to take any teaching from Clerc, and no motivation to choose a particular internal/external medium from alternatives suggested in the portion indicated by the Examiner.

49. Forssen has increased storage period of loaded liposomes to 20 weeks by using a negatively charged lipid, much more than what Janoff has taught by using sucrose. Forssen notes that "Although methods for making liposomes are well known in the art, it is not always possible to determine a working formulation without undue experimentation."
50. In my opinion, Hong is not enabling, and as such, no liposomes made by the Hong method have been seen in the market place. In contrast, the instant invention process is enabling and has been successfully commercialized as noted in paragraph 5 above. The present invention is clearly not obvious. If the process of the instant invention was so obvious, why did not somebody think of it before the present application when all the prior art documents cited were already known years before the instant invention? Not until the present inventors invented the claimed process (which was 13 years after Janoff and 22 years after Papahadjopoulos) did someone think of adding sucrose to the hydration medium, and/or maintain volume of hydration medium and/or to use histidine sucrose medium to remove extraliposomal ammonium sulphate in dialysis medium, as in instant claim 1, to get a liposome with increased circulation time without the use of PEG.
51. To summarize, the combinations of references cited in the Office Action are listed in TABLE II below. Three combinations are separately given and in each of them, the

combination of one reference with the primary reference is given in the first column. The second column provides for what purpose this combination is given and the third column provides why the reasoning given for such combination is not persuasive. It will be seen that the reasoning for the suggested combinations is not persuasive. Further, the objectives of the points put forth in these combinations are limited to making up certain differences from the instant invention in the primary reference chosen as close prior art, but the synergistic action is not limited only to these differences. That is, important considerations such as the many other differences in the process steps, composition details and quantitative aspects which are not obvious from these references is not provided. Hence the instant invention is not obvious from any of these three combinations.

TABLE II: Combination of references

Combination 1: Hong, Papahadjopoulos, Janoff and optionally Barenholz

Two combined	Combined for	Reason why combination is not persuasive
Hong + Papahadjopoulos	1. Volume of hydration medium	<p>a) The process of hydration is different from that in Hong, and it is not compatible with that in Hong. Papahadjopoulos process is a two stage process, it goes through a gel stage, So also hydration in presence of solvent is not compatible with process of Hong.(Hong example 2).</p> <p>b) volume of the hydration media is outside to that in instant claim 1, which is for mMole of phospholipid and not per total lipid. This volume in both examples 1 and 2 is not 1.5 ml, it is 1.5 ml twice one before gel formation and another after gel formation when liposome is formed.</p> <p>c) hydration medium and phospholipids used in the two processes are different and are different from instant claim 1 (Lipids contain phosphatidylglycerol).</p> <p>d) Objective of Papahadjopoulos is to increase circulation time using lipid polymer conjugate. (para. 33, 34)</p>

	2. Inclusion of histidine in hydration buffer	2. This is not relevant, instant claim 1 does not use histidine in hydration buffer (para. 39).
Hong + Janoff	1.Sucrose inside and outside liposome retains Adriyamycin during dehydration and rehydration	<p>a) There is no knowledge before this application that presence of sucrose would increase circulation period. (para. 28).</p> <p>b) In Janoff sucrose with HEPES and NaCl is inside liposome and not just sucrose. Separating sucrose from HEPES and NaCl and combining it with ammonium sulphate in Hong is not obvious. (para. 30).</p> <p>c) Janoff teaches dehydration and rehydration of liposomes and not liposomes with longer circulation time. (para. 28).</p> <p>d) Janoff's stability is tested of dehydrated liposomes (moisture less than 2%) stored in refrigerator up to 7 days and there is no indication whether it will stand in aqueous conditions while circulating in blood at body temperature. (See, TABLE I and para. 27).</p>
	2.Volume of hydration media	1. Lipid (EPC) used in Janoff is different, hydration medium used is different from Hong and instant claim 1, volume of hydration medium, being dependent on these factors, is not relevant for applying to Hong hydration medium with any expectation of success in raising circulation period. (para-35)
Hong + Barenholz	Removal of outside ammonium sulphate	<p>Barenholz does not have the step of removal of extraliposomal ammonium sulphate by dialysis using sucrose-histidine buffer, and it cannot make this step obvious.</p> <p>a) Neither Hong nor Barenholz teach removal of ammonium sulphate by dialysis using sucrose histidine buffer as taught in instant claim 1. And there is no evidence to believe that that such a step would contribute, along with other features of the instant invention, to raising circulation rate of the liposome formed, without the gleanings from the present claim 1. (para 31).</p> <p>b) In the present Invention, it is not removing just ammonium sulphate from</p>

		the extraliposomal media but while doing so it also improves in parts the desired structure to the liposome with right permeability of the liposomal membranes to give longer circulation period and deliver the drug slowly over the extended period of circulation. (See TABLE I and para 27.)
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Combination 2: Slater and Janoff

Two references combined	Combined for	Reason why combination is not persuasive
Slater + Janoff	1.Having sucrose in hydration buffer	1. As in Hong and Janoff in Combination I above.
	2. hydration volume	2. Lipid (EPC) used in Janoff is different, hydration medium used is different from Slater and instant claim 1, volume of hydration medium being dependent on these factors is not relevant for applying to Slater hydration medium with any expectation of success in raising circulation period. (See TABLE I and para 23).

Combination 3: Forssen, Janoff, Papahadjopoulos, optionally Slater and/or Clerc.

Two references combined	Combined for	Reason why combination is not persuasive
Forssen + Janoff	1.Having sucrose in Hydration medium:	1. As in Hong and Janoff in Combination I above.
	2.hydration volume	2. Lipid (EPC) used in Janoff is different, hydration medium used is different from Forssen and instant claim 1, hence volume of hydration medium, being dependent on these factors, is not relevant for applying to Forssen hydration medium with any expectation of success in raising circulation period. (See TABLE I and para 23).
Forssen + Papahadjopoulos	1.Volume of hydration medium	1. as in Hong and Papahadjopoulos in Combination 1 above

	2. Inclusion of histidine in hydration buffer	2. This is not relevant, instant claim 1 does not use histidine in hydration buffer (See TABLE I and para. 39).
Forssen + Slater	Removal of outside ammonium sulphate	Slater does not make the step of removal of extralposomal ammonium sulphate by dialysis using sucrose-histidine buffer, obvious, because Slater does not suggest that a) Forssen does not teach use of sucrose histidine buffer for removal of ammonium sulphate. (See TABLE I and para. 42.)
Forssen + Slater +/or Clerc	Composition of hydration buffer	Forssen + Slater as alternative to show obviousness is not persuasive as said above. Clerc does not teach hydration medium composition as in Claim 1 of the present invention. (See TABLE I).

52. I furthermore declare that that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardized the validity of the application or any patent issued thereon.

29/08/2011

Date

Dr. Gautam Vinod Daftary

Annexure 1**List of Patent Applications**

1. Liquid Stable Composition of Oxazaphosphorine with Mesna (WO2004/022699)
2. Ifosfamide Compositions for Parenteral Administration and a Process for their Preparation (WO2004/050012)
3. Non-pegylated Long-circulating Liposomes (WO2004/058140)
4. Stable Emulsion Compositions for Intravenous Administration having Preservative Efficacy (WO2006/030450)
5. Intravenous Propofol Emulsion Compositions having Preservative Efficacy (WO2007/052288)
6. Aqueous Anaesthetic Compositions Comprising Propofol (WO2007/052295)
7. Propofol Emulsion Compositions for Intravenous Administration (WO2008/023384)
8. Stable Injectable Oil-in-Water Docetaxel Nanoemulsion (WO2010/018596)
9. Anti-RhD Monoclonal Antibodies (WO2010/079510)